

Development of a K562 cell-based assay for screening anticancer agents¹

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KEY WORDS antitumor drug screening assays; K562 cells; cell culture; drug screening; antineoplastic agents

ABSTRACT

AIM: To develop a leukemia cell line K562-based assay for high-throughput screening. **METHODS:** The screening was carried out on 96-well plates with monitoring cell proliferation by a combined 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium (MTS)/phenazine methosulfate (PMS) method. Conditions for evaluating effects on the proliferation of K562 cells by individual compounds on the 96-well plates were optimized. **RESULTS:** A set of 800 small organic compounds was screened for anticancer activity by this cell-based assay, with consumption of each compound at 500 ng. Eleven compounds were identified with >80% inhibitory activity at 5 mg/L, among which 9 compounds were confirmed by subsequent testing at multiple concentrations. The most potent compound showed an IC₅₀ at 170 nmol/L, and there were total of 7 compounds showed IC₅₀ less than 10 μmol/L. **CONCLUSION:** The high-throughput method using K562 cell line is fast, economical, effective, and practical in identifying inhibitors as potential therapeutic agents for cancer.

INTRODUCTION

Cancer is the major health concern, and current cancer therapies include chemotherapy, radiation therapy,

and surgery. Although there are a few drugs showing effectiveness in treating certain cancers, but their side effects and toxicity call for new and more effective anticancer agents.

Various cancer cell lines have been used for screening compounds for anticancer activity. National Institutes of Health (NIH) USA used panels of 60 cell lines since early 1990's^[1], and recently reduced to 3 cell lines prescreen followed by 60 cell lines, five dose testing if it meets certain activity criteria. Apparently, it is costly to carry out such screening, and it becomes prohibitive in large scale screening of samples from combinatorial synthesis and natural product isolation. There are needs to develop more efficient and cost-effective cell-based assay.

We have been developing assays suitable for high-throughput screening (HTS) for identifying compounds with anticancer activity using the various cancer cell lines and molecular targets involved in cancer metastasis and angiogenesis^[2]. Leukemia cell line K562 grows in suspension and easy to monitor its growth on a microtiter plate, and it is one of the cell lines that can be adapted for HTS assays. We report here a simple and cost-effective 96-well plate-based assay using K562 cells for screening anticancer agents.

MATERIALS AND METHODS

Materials and instruments The cell line K562 derived from human chronic myelogenous leukemia was purchased from American Type Culture Collections (Manassas, VA, USA; ATCC number CCL-243). Culture medium RPMI-1640 and newborn calf serum was purchased from Hyclone (Logan, UT, USA). 3-[4,5-Dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Phenazine methosulfate (PMS) was the product of Sigma (St Louis, MO, USA). Other reagents and solvents used in

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the experiments were of either analytic grade or reagent grade as appropriate.

Samples used in the cell-based assay were all small organic compounds with purity higher than 90 %, either purchased or collected from different sources with wide structural diversity.

Biomek 2000 liquid handling workstation from Beckman (Fullerton, CA, USA) and eight-channel pipette from Biohit (Helsinki, Finland) were used for liquid handling, and SPECTRAMax 340 96-well plate reader from Molecular Devices (Sunnyvale, CA, USA) was used for colorimetric end point readings. The 96-well plates used for cell culture were the products of Corning Costar (Stony Brook, NY, USA).

Culture conditions for K562 cells K562 cells were cultured in 25-cm² flasks as suspension in the RPMI-1640 medium. After centrifugation at 150 × *g* for 10 min, the collected cells were separated into single-cell suspensions by a gentle pipetting action, and then counted using trypan-blue exclusion on a hemacytometer. Viability of the cells greater than 98 % as determined by trypan-blue exclusion was checked routinely, and the cells were diluted to give a density of 2.5 × 10⁷/L in RPMI-1640 containing 5 % newborn calf serum. The cells in the presence or absence of inhibitors were incubated at 37 °C in a humidified 5 % CO₂ atmosphere.

Assay for K562 proliferation A method using MTS^(3,4) was adapted for monitoring the cell proliferation on 96-well plates. In principle, dehydrogenase found in metabolically active cells converts MTS into the aqueous soluble formazan, which is determined by absorbance difference between 490 nm and 690 nm. A combined MTS/PMS solution (MTS 2 g/L and PMS 46 mg/L in Dulbecco's phosphate buffered saline, 25 μL) was added manually to each well with viable cells. The plates were incubated for 4 h at 37 °C in the humidified 5 % CO₂ atmosphere, and the absorbance difference was determined using the SPECTRAMax 340 plate reader. The absorbance differences were converted to numbers of viable cells according to a standard curve generated with counted cells (Fig 1).

Sample preparation Individual compounds (1 mg each) were dissolved in 200 μL of Me₂SO as 5 g/L solutions. The solution was diluted 10 folds by adding 10 μL of the solution to 90 μL of Me₂SO in the sample wells (A2-H11) of 96-well polypropylene plates as mother plates (Fig 2), and the concentration was 500 mg/L for each compounds. Each mother plate was duplicated to polystyreneplates as daughter plates for

screening by transferring 1 μL of the solution using the Biomek 2000 workstation. The final concentration of the compounds was 5 mg/L after adding 100 μL cells in culture medium.

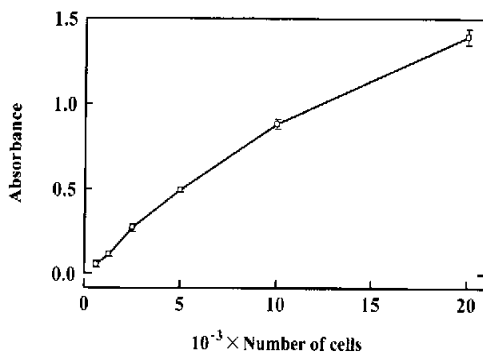


Fig 1. Correlation between the number of viable K562 cells and the absorbance difference between 490 nm and 690 nm. *n* = 3. $\bar{x} \pm s$.

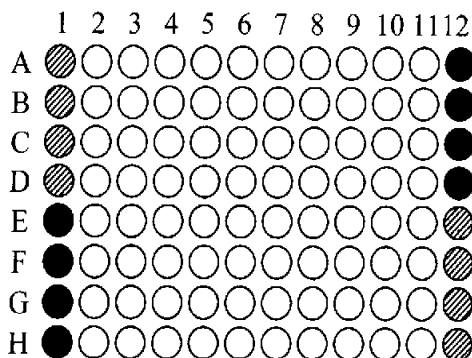


Fig 2. Layout of samples (open circles), blanks (shaded circles), and controls (filled circles) on the 96-well plate. Rows A to H and columns 1 to 12 are shown.

K562 cell-based high-throughput screening (HTS) assay On the daughter plates with the same layout as the mother plates (Fig 2), Me₂SO solvent (1 μL) were distributed in eight blank wells (A1-D1, E12-H12), and controls were in other eight wells (E1-H1, A12-D12) using known active compound etoposide in Me₂SO (1 μL; 10 mmol/L, 1 mmol/L, 100 μmol/L, and 10 μmol/L).

The K562 cells (100 μL, 2.5 × 10⁷/L in RPMI-1640 with newborn calf serum) were distributed to each well on the 96-well plates with an 8-channel pipette, and were incubated at 37 °C in the humidified 5 % CO₂ atmosphere for 48 h. The MTS/PMS solution (25 μL)

was added with eight-channel pipette to each well, and the UV absorbance difference between 490 nm and 690 nm was determined after 4 h incubation.

A background value, which was averaged from wells without cells on a separated plate, was subtracted from the values of wells with cells. The averaged value from the 8 blanks was used as 100 % cell growth. The value from control wells or compound wells was divided by the average, giving percent cell growth in the presence of the active compound etoposide or the screening compounds (Fig 3).

RESULTS

Defining the conditions for the cell-based assay Since Me₂SO solvent was used in dissolving compounds for the cell-based assay, we first investigated the effects of Me₂SO on the growth of K562 cells. The cells (inoculation density at 2.5 × 10⁷/L) were incubated with different amounts of Me₂SO for 48 h, and the growth of cells in each well were determined by the MTS method. As increase of Me₂SO concentration, the growth of K562 cells slowed down accordingly. The K562 cells showed 86 % ± 8 % growth at 0.5 % Me₂SO, 82 % ± 2 % growth at 1 % Me₂SO, 77 % ± 3 % growth at 2 % Me₂SO, and 55 % ± 3 % growth at 4 % Me₂SO, comparing with no Me₂SO as 100 % growth. The data suggested that presence of 1 % Me₂SO in the cell-based assay did not markedly affect the cell growth.

The MTS method used under our assay condition showed good correlation between absorbance reading at 490 nm and the actual cell numbers, and we saw a linear relationship up to the cell density at 2 × 10⁸/L (Fig 1). For an accurate reading after 48 h incubation, we hope that the cell density will reach a level still within the

linear range of the MTS assay. We inoculated cells on the 96-well plates at several cell densities and measured the absorbance readings after 1 – 4 d incubation. We found that the inoculation cell density at 2.5 × 10⁷/L gave responsive and reproducible growth curve (Fig 4), which was used in our cell-based assay.

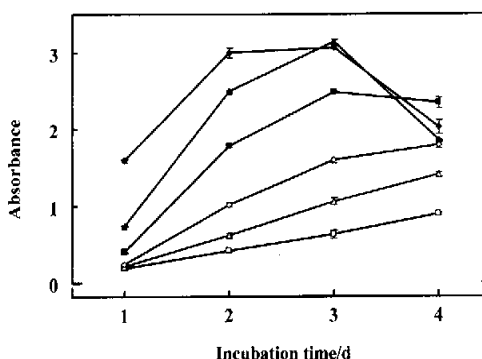


Fig 4. Cell growth curves after incubation of 1 to 4 d as determined by absorbance differences between 490 nm and 690 nm. Cell densities were 6.25 × 10⁶/L (□), 1.25 × 10⁷/L (△), 2.5 × 10⁷/L (◇), 50 × 10⁷/L (■), 1 × 10⁸/L (▲), and 2 × 10⁸/L (◆). n = 3. x ± s.

After initial experiments mentioned above, we defined the assay condition as final concentrations at 2.5 × 10⁷/L K562 cells in RPMI-1640 medium with 5 % newborn calf serum, 1 % Me₂SO, and screening compounds 5 mg/L in a total volume of 100 μL. Me₂SO solvent was used as blanks, and etoposide in Me₂SO at final concentrations of 100, 10, 1, and 0.1 μmol/L were used as controls.

Screening of 800 compounds by the K562 cell-based assay The total of 800 compounds was accommodated on ten 96-well plates. Compounds with no effect on the growth of cells showed the values of UV

	1	2	3	4	5	6	7	8	9	10	11	12
A	101	80	92	94	94	93	57	96	86	88	94	18
B	99	78	90	84	41	29	88	92	82	86	77	29
C	96	92	60	92	100	103	99	97	55	69	86	56
D	103	104	78	69	99	98	78	70	96	76	89	85
E	83	93	93	53	89	89	83	91	85	88	83	98
F	59	88	88	98	99	64	85	82	69	96	72	101
G	33	79	85	96	91	89	95	46	80	35	14	102
H	16	80	95	83	19	64	63	72	92	88	69	99

Fig 3. Cell growth as determined by the MTS method. Percent cell growths in each well of one typical 96-well plate are shown.

absorption similar to the blanks, while those compounds with cell toxicity or growth inhibition changed the value to a reduced number by different degrees, reflecting their inhibitory potency on cells growth (Fig 3). We obtained percent cell growth in each compound and control well by dividing the value in the compound and control wells by that in the eight blank wells.

As expected, most compounds showed no or weak growth inhibition. There were 52 compounds showed 40 % - 60 % inhibition, 25 compounds showed 60 % - 80 % inhibition, 11 compounds showed inhibition higher than 80 %, and the rest showed no inhibition or less than 20 % inhibition at the assay concentration of 5 mg/L. We took the 11 compounds with inhibition higher than 80 % as hits for further characterization, and the hit rate was 1.4 % of the total 800 compounds.

Quality controls in the HTS assay In order to handle a large number of compounds in a HTS assay and manage huge amount of information generated, it is the common practice to assay compounds only at a single empirical concentration with hope to identify hits for further investigation. To differentiate fluctuations due to

liquid handling and instrument reading from real inhibition, we tested the precision in pipetting and instrument reading in situations we used for our HTS assays.

We first tested the precision of pipetting by Biomek 2000 liquid handling workstation, using the dye Bromophenyl Blue (BPB) as the indicator and measuring absorbance at 592 nm on 96-well plates. When 1 μ L of BPB in Me₂SO was added by Biomek 2000 to dry plates, simulating compound distribution to daughter plates, the relative standard deviation (RSD) was 9 % across the entire plate (Fig 5). This is consistent with our previous results, in which 2 μ L of BPB in Me₂SO was transferred to dry plates, and RSD was 7 %^[2].

We replaced compounds in sample wells and control wells with Me₂SO solvent and measured cell growth after 48 h incubation (Fig 5). The variation should reflect the combined effect of pipetting, cell growth, as well as formazan measurement, and we found that the cell growth on the entire plate was quite uniform with RSD at 9 %.

We used the known active compound etoposide as the control at four final concentrations from 100 to 0.1 μ mol/L. Consistently, etoposide showed K562 growth

A	1	2	3	4	5	6	7	8	9	10	11	12		
A	1.01	1.01	1.02	1.01	1.02	1.02	1.02	1.02	1.03	1.00	1.02	1.00	1.01	<i>0.01</i>
B	1.03	0.98	1.07	1.08	0.96	1.03	1.03	1.04	1.01	0.99	1.02	1.01	1.02	<i>0.03</i>
C	1.04	1.03	1.05	1.00	1.07	1.36	1.04	1.32	1.07	1.28	1.04	1.18	1.12	<i>0.11</i>
D	1.03	1.03	1.06	1.17	1.13	1.10	1.07	1.05	1.08	1.01	0.97	1.00	1.06	<i>0.05</i>
E	1.02	1.02	1.03	0.97	0.96	0.97	0.98	0.96	0.96	0.99	0.95	1.24	1.00	<i>0.08</i>
F	1.01	1.02	1.03	1.03	1.00	1.29	1.08	1.23	1.02	1.01	0.99	1.25	1.08	<i>0.10</i>
G	1.01	1.01	1.01	1.01	0.99	1.14	1.00	1.17	0.99	1.27	1.27	1.10	1.08	<i>0.10</i>
H	1.03	1.02	1.03	1.03	1.02	1.01	1.02	1.23	0.98	1.36	1.01	1.00	1.06	<i>0.11</i>
	1.02	1.02	1.03	1.04	1.02	1.12	1.03	1.13	1.02	1.11	1.03	1.10	1.05	
	<i>0.01</i>	<i>0.02</i>	<i>0.02</i>	<i>0.06</i>	<i>0.06</i>	<i>0.13</i>	<i>0.03</i>	<i>0.11</i>	<i>0.04</i>	<i>0.14</i>	<i>0.10</i>	<i>0.10</i>		<i>0.09</i>
B	1	2	3	4	5	6	7	8	9	10	11	12		
A	1.24	1.21	1.25	1.29	1.27	1.27	1.29	1.36	1.25	1.23	1.27	1.30	1.27	<i>0.03</i>
B	1.24	1.17	1.27	1.30	1.29	1.28	1.41	1.41	1.42	1.29	1.29	1.24	1.30	<i>0.06</i>
C	1.41	1.24	1.36	1.30	1.28	1.28	1.34	1.29	1.27	1.19	1.26	1.22	1.29	<i>0.05</i>
D	1.44	1.43	1.43	1.33	1.32	1.38	1.42	1.40	1.40	1.36	1.36	1.36	1.39	<i>0.03</i>
E	1.43	1.35	1.43	1.55	1.42	1.52	1.52	1.52	1.50	1.45	1.40	1.39	1.46	<i>0.04</i>
F	1.29	1.28	1.39	1.37	1.39	1.44	1.37	1.43	1.47	1.28	1.42	1.24	1.36	<i>0.05</i>
G	1.49	1.56	1.47	1.53	1.59	1.26	1.52	1.15	1.41	1.41	1.39	1.37	1.43	<i>0.09</i>
H	1.28	1.18	1.20	1.22	1.21	1.16	1.08	1.05	1.08	1.10	1.03	1.10	1.14	<i>0.07</i>
	1.35	1.30	1.35	1.36	1.34	1.33	1.37	1.33	1.35	1.29	1.30	1.28	1.33	
	<i>0.07</i>	<i>0.11</i>	<i>0.07</i>	<i>0.09</i>	<i>0.09</i>	<i>0.09</i>	<i>0.10</i>	<i>0.12</i>	<i>0.10</i>	<i>0.09</i>	<i>0.10</i>	<i>0.08</i>		<i>0.09</i>

Fig 5. (A) Variation from pipetting 1 μ L Me₂SO to dry plate. (B) Variation in cell growth as measured by MTS method. The averages of rows, columns, or entire plates were shown as bold face, and the RSD's of rows, columns, and plates as italics.

inhibition with the expected potency ($86\% \pm 3\%$ growth inhibition at $100\ \mu\text{mol/L}$, $69\% \pm 3\%$ growth inhibition at $10\ \mu\text{mol/L}$, $41\% \pm 4\%$ growth inhibition at $1\ \mu\text{mol/L}$, and $13\% \pm 4\%$ growth inhibition at $100\ \text{nmol/L}$).

With these tests on precision of pipetting and instrument reading, as well as data from the control compound etoposide, we understand the degree of fluctuation due to pipetting and reading, and we can comfortably conclude that we would most likely see an inhibition if the compound to be screened has significant effect on the proliferation of the K562 cells.

Characterization of the hit compounds The 11 compounds showed inhibition higher than 80% were further investigated. At first, we checked their inhibition at eight final concentrations in 10 fold differences from $50\ \text{mg/L}$ to $5\ \text{ng/L}$. Two compounds did not show the expected inhibition and were considered false positives. The rest showed significant inhibition at concentrations lower than $5\ \text{mg/L}$. Then we chose concentrations at 50 , 15.6 , 5 , 2.8 , and $1.5\ \text{mg/L}$, 860 , 500 , $50\ \mu\text{g/L}$ to determine their IC_{50} values which were calculated by Sigmaplot software. Among those tested, 9 compounds showed IC_{50} lower than $5\ \text{mg/L}$.

After taking their molecular weights into account for each active compound, the best compound SH00010034 obtained from this screening showed an IC_{50} at $170\ \text{nmol/L}$, followed by those with IC_{50} at $700\ \text{nmol/L}$, 3.5 , 3.9 , 4.8 , 6.4 , $6.5\ \mu\text{mol/L}$. There were total 7 compounds with IC_{50} lower than $10\ \mu\text{mol/L}$, and 2 compounds with IC_{50} between 10 and $20\ \mu\text{mol/L}$, demonstrating the power of the HTS assay in identifying K562 growth inhibitors. These compounds are under further structure-activity analysis and structural modifications.

DISCUSSION

We have developed a K562 cell-based assay on 96-well plates, monitoring cell proliferation by MTS method. The assay is simple, reliable, and cost-effective. Only $500\ \text{ng}$ of compound each was used for the screening at one concentration, giving statistically significant potency values. A set of 800 compounds were screened using the assay, and a few active compounds were identified with the best one with IC_{50} at $170\ \text{nmol/L}$, offering leads for further structural improvements.

NIH used a panel of 60 cancer cell lines for

evaluation, and each determined at several concentrations⁽¹⁾. Although it was effective and reliable, it was costly especially for large-scale screening. With careful control of assay conditions, we demonstrated that it was possible to screen compounds at only one single concentration and obtain statistically meaningful results. Adding the costs for culture medium, reagents, and disposables used in the screening, per-well cost for the screening is well under USD \$ 1, promising it as a high throughput format for large scale screening for anticancer agents.

Currently, there are several methods used most frequently for monitoring cell proliferation, including MTT⁽⁵⁾, XTT⁽⁶⁾, and SRB⁽⁷⁾. We chose the recently developed MTS method^(3,4) because its MTS product formazan is soluble in the culture media, so that we can perform the assay with no washing or cell harvesting steps, facilitating the partially automated HTS assays.

Several cell lines should be adaptable to HTS, and the K562 cell-based assay should be applicable to other cells growing in suspension. We are currently working on conditions for other cells including adherent cells and applying these methods for screening for anticancer agents.

REFERENCES

- 1 Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, *et al.* Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991; 83: 757-66.
- 2 Ye QZ, Nan FJ, Hu LY, Qian Z, Qian J. High-throughput screening for human collagenase 1 inhibitors. *Acta Pharmacol Sin* 2000; 21: 1021-6.
- 3 Barltrop JA, Owen TC, Cory AH, Cory JG. 5-(3-Carboxyl-methoxyphenyl)-2-(4, 5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of MTT reducing to purple water-soluble formazans as cell-viability indicators. *Bioorg Med Chem Lett* 1991; 1: 611-4.
- 4 Goodwin CJ, Holt SJ, Downs S, Marshall NJ. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. *J Immunol Methods* 1995; 179: 95-103.
- 5 Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, *et al.* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 1988; 48: 589-601.
- 6 Roehm NW, Rogers GH, Hatfield SM, Galsebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J Immunol Methods* 1991; 142: 257-65.
- 7 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J,

Vistica D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; 82: 1107-12.

以 K562 细胞为靶点的抗肿瘤药物筛选模型的建立¹

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关键词 抗肿瘤药物筛选试验; K562 细胞; 细胞培养; 药物筛选; 抗肿瘤药

目的: 建立以白血病细胞系 K562 为靶点的高通量抗

肿瘤药物筛选模型。 **方法:** 在 96 孔细胞培养板上, 运用四唑氮化合物(MTS)和电子耦联剂(PMS)连用的方法, 对 K562 细胞增殖情况进行检测。对在化合物影响下 K562 细胞增殖变化的检测条件进行了优化。 **结果:** 采用这一细胞水平的高通量抗肿瘤药物筛选模型, 完成了 800 个小分子有机化合物的筛选, 每个化合物的用量是 500 ng。11 个化合物在浓度为 5 mg/L 时可抑制细胞增殖达 80 % 以上, 其中 9 个通过多浓度复筛得到了确认。抑制活性最强的化合物的 IC₅₀ 为 170 nmol/L, 共有 7 个化合物显示 IC₅₀ 低于 10 μmol/L。 **结论:** 采用 K562 细胞系进行高通量筛选是快速、经济、有效、实用的发现新型抗肿瘤药物的方法。

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